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CLONING, CHARACTERIZATION AND EXPRESSION OF CHICKEN (GALLUS GALLUS LINNAEUS) BONE MORPHOGENIC PROTEIN 2 IN PICHIA PASTORIS

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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October 2015

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Bone Morphogenetic Protein 2 (BMP2) is well known with its significant role in bone healing process and oesteogenesis. It was accidentally discovered by Urist in 1965, when he implanted a demineralized bone in the muscle pouch of a rat. Sampath and Reddi (1981) had further confirmed the effect and characteristic of BMP2 protein. Whereby, when BMP2 protein was removed from the matrix, the matrix failed to induce new bone formation and vice versa. Hence, the effect of Recombinant BMP2 has been tested on most of the mammalian host such as, sheep, rabbits, rats and dogs but not much was done on avian system. Therefore, the aim of this study is to detect the presence of BMP2 gene within the avian genome by using PCR-based method. Primers that targeted BMP2 gene within the avian genome was designed based on the gene sequence from National Centre for Biotechnology Information (NCBI). DNA from domesticated chicken was extracted from the primary feathers and further subjected to Polymerase Chain Reaction (PCR) screening. The PCR results showed positive results for BMP 2 gene detection within the avian genome and the PCR results were sent for sequencing. Subsequently, the sequencing results were used to BLAST against the NCBI gene data bank. The sequencing results returned with successful amplification of BMP2 gene from *Gallus gallus*. The original BMP2 sequence (NM_204358) was then translated into amino acid sequence and sent for codon optimization via codon substitution. Research also has shown that, codon optimization not only resulted in better gene expression but at the same time the expression products could be purified more readily as compared to the native version. Therefore, with gene synthesis method another new nucleotide sequence of BMP2 was synthesized and it consisted of low % of GC content as compared to the native BMP2 gene and had better stability during the expression stage. The new BMP2 gene was then cloned into *Pichia* pink α-HC plasmid and relabeled as BMP2v2. The reduction of GC% also had direct impact on the annealing temperature of the primers set from 71.6^{o}C reduced to 63^{o}C and non-specific binding as well. The chromogenic results also had further confirmed the presence of BMP2 protein. Therefore, BMP2 protein was successfully cloned and expressed in *Pichia pastoris*. Whereby, this BMP2 protein could be further applied in clinical application in avian system in the future.

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PENGKLONAN, PENCIRIAN DAN EKSPRESI PROTEIN MORFOGENETIK TULANG 2 AYAM (*Gallus gallus* Linnaeus) DI DALAM *Pichia pastoris*

Oleh

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Oktober 2015

Pengerusi : Profesor Madya Jalila Abu, PhD
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Protein morfogenetik tulang 2 (BMP2) yang dikenali dengan peranannya yang penting dalam proses penyembuhan tulang dan oesteogenesis. Ianya secara tidak disengaja ditemui oleh Urist pada 1965 ketika beliau implan tulang dimineral pada otot tikus. Sampath dan Reddi (1981) telah mengenalpasti kesan dan pencirian protein BMP2. Apabila protein BMP2 ini diasingkan daripada matriks, tulang yang baru gagal dibentuk dan sebaliknya. Oleh itu, kesan protein BMP 2 rekombinan telah dikaji ke atas kebanyakan mamalia seperti kambing bebiri, arnab, tikus dan anjing tetapi tidak banyak kajian dijalankan ke atas burung. Oleh itu, matlamat kajian ini adalah untuk mengesan kehadiran gen BMP2 di dalam genom burung dengan penggunaan kaedah berasaskan PCR. Primer-primer yang mensasarkan gen BMP2 di dalam genom burung telah direka bentuk berdasarkan urutan gen dari National Centre for Biotechnology Information (NCBI). DNA dari bulu ayam diekstrak dan diperiksa oleh Polymerase Chain Reaction (PCR). Hasil PCR menunjukkan pengesanan positif untuk kehadiran gen BMP2 dalam genom burung dan telah dihantar untuk penghasilan urutan. Kemudiannya, hasil urutan telah digunakan untuk BLAST menggunakan data dari genbank NCBI. Hasil urutan kembali dengan kejayaan amplifikasi gen BMP 2 dari *Gallus gallus*. Urutan asal BMP2 (NM_204358) kemudiannya dijemahkan kepada urutan asid amino serta dihantar untuk pengoptimuman kodon melalui penggantian kodon. Penyelidikan menunjukkan bahawa pengoptimuman kodon bukan sahaja menghasilkan ekspresi gen yang lebih baik malah pada masa yang sama produk yang diekspresi boleh dipurifikasi lebih mudah berbanding dengan versi asli. Oleh itu, melalui kaedah sintesis gen, urutan nukleotida baru BMP2 telah dihasilkan dan ianya mengandungi peratusan GC yang rendah berbanding dengan gen BMP2 yang asli dan ia adalah lebih stabil ketika peringkat ekspresi. Gen BMP2 yang baru ini kemudiannya diklon ke dalam plasmid α-HC *Pichia* pink dan dilabel semula sebagai BMP2v2. Pengurangan %GC juga mempunyai impak secara langsung ke atas suhu pelekatan primer-primer yang ditetapkan pada 71.6^{o}C dikurangkan kepada 63^{o}C dan juga pengikatan tidak khusus. Keputusan kromogenik juga mengesahkan secara lanjut kehadiran protein BMP2. Oleh itu, protein BMP2 telah berjaya diklon dan diekspresi dalam *Pichia pastoris*. Protein BMP2 ini boleh digunakan dalam aplikasi klinikal di dalam sistem avian pada masa hadapan.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;

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LIST OF ABBREVIATIONS

AOX	Aldehyde oxidase
AP	Alkaline phosphatase
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol-complex medium
BMP	Bone morphogenetic rotein
CHO	Chinese hamster ovary
DBM	Demineralized bone matrix
ddH_2O	Double distilled, deionized water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotides
E.coli	*Escherichia coli*
g	gravity force unit
HCl	Hydro chloride acid
HEK	Human embryonic kidney
Hela	Human cervical cancer
HRP	Horseradish peroxidase
K	1000
Kbps	Kilo base pairs
LB	Luria broth
LPS	Lipopolysaccharide
mg	Miligram
µL	Microlitre
µm	Micrometer
$MgCl_2$	Magnesium chloride
ML	Mililitre
mM	Millimolar
mRNA	Messenger RNA
NCBI	National Centre for Biotechnology Information
^oC	Degree celsius
OD	Optical density
PAD	*Pichia* adenine dropout
PCR	Polymerase chain reaction
rhBMP	Recombinant human bone morphogenetic protein
RNA	Ribonucleic acid
Rpm	Revelution per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide
TAE	Tri-acetate-EDTA buffer
Taq	Thermus aquaticus
TGF-B	Transforming growth factor beta
V	Volatage
w/v	Weight/volume
YPD	Yeast peptone dextrose
YPDS	Yeast peptone dextrose sorbitol

CHAPTER 1

INTRODUCTION

Bone fracture is one of the most common issues found on both wild and captive birds according to Coles (2008); Kubiak and Forbes (2011). Most of the time such injuries leads to death as proper treatment is not available and at the same time the recovery rate of the fracture wound is often poor with high incidence of complication (Rinkevich et al. 1999). On top of that, Bennett (1992) also comment that, the avian bones have high content of calcium as compared to mammal bones and that make it more prone to shatter upon high impact. At the same time, based on an annual observation reported by Janelle (2002), between year 1994 to 1998 in Mexico alone there were total of 265 cases of wild bird mortalities reported merely due to bone fracture caused by instant crash landing, panicky flight and downed by storm Hamilton (2007). These bird mortalities made up 23% of mortality rate in Mexico within these five years.

According to Brown (2009) and Rinkevich et al. (1999), bone fracture healing processes involves complex process of cell proliferation and differentiation. It is also a highly specific process as anything less than complete normal function cannot be regarded as complete healing process, especially fracture wound that occur at humerus bone. A slightly changes in degree of rotation can result in a severe loss of flight function during the healing process.

Apart from that, the time length of the entire healing process is also subjective to each bird. This factor leads to the increase in bird mortalities rate especially for nomadic bird species, as longer rehabilitation time will prevent healed birds from reuniting with their flocks and this will cause depression and often lead to death of these birds (Brown 2009).

During the healing process, growth factors (BMP group), inflammatory cytokines, antioxidants, osteoblast, osteoclast, amino acids and nutrients will play their role together to signal initiation of cell differentiation and proliferation at the fracture site. The first stage of the healing process (inflammation phase) happens when bone fracture occur (Brown 2009). At this phase, blood clot will form, allowing the influx of inflammatory, clean-up cells to the wound area, followed by cytokine cascade, which will signal the repair cells to the fracture site.

Next, they begin to differentiate into specialize cells that build new bone tissue and new cartilage. Both osteoblasts and chondroblasts cells will begin the repair process, lying down new bone matrix and cartilage (Brown 2009). Subsequently the cells will undergo cell breakdown process, which known as osteoclast.

The second stage of bone healing is reparative stage, whereby the protein produced by the osteoblasts and new cartilage will form soft callus and begin to harden, forming

hard callus. The final stage of bone healing (remodeling stage), involves the callus maturing, and remodeling itself. Woven bone will then remodeled into stronger lamellar bone by the combined action of both osteoblast bone formation and osteoclast bone resorption cells. This entire process will take about three to four months (Brown 2009). Many researches and clinical trials had proven that Bone Morphogenetic Protein (BMP) group is a type of multifunctional growth factor that has many roles in the body. Bone Morphogenetic Protein will act together with different types of chemical signaling within the body and express its different effects at the respective parts of body when needed. Such chemical signaling effects include development of both central and peripheral nervous system in vertebrates (Liu and Lee 2005), cardiac cells differentiation (Van et al. 2006), bone and cartilage formation as well as bone healing on fractured bone (Setti 2001). Among known BMP group that exhibit such effect were BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8, BMP9, BMP10 and BMP15 (Yong et al. 2007).

Among the listed BMP groups, BMP2 is currently been widely studied on its role in bone formation and it was proven that BMP2 can induce bone formation in mammals (Nilsson et al. 1986; Wang et al. 1990; Bouxsein et al.2001; Scott et al. 2002; Ulmanen et al. 2005). Apart from that, Harvinder (2000) had also been using BMP2 to treat long bones fractures and spinal fusion procedures. In 2005, Toshiyuki et al. had successfully shown that osteocyte-like cells and osteoblast-like cells were observed after 14 days of implantation of 5µg/ml of recombinant human Bone Morphogeneic Protein 2 (rhBMP2) into the rats' calf muscle.

According to Yoshida et al. (1998), even a small amount as 5µg/ml of BMP2 is sufficient to induce new bone formation in the subcutaneous tissue. Therefore, BMP2 had shown some significant effects in inducing new bone formation especially in some fracture gap studies. Sample et al. 2008 successfully used recombinant human BMP2 and mixed with calcium phosphate matrix paste to treat a 3.5 month old whooping crane with open humeral fractures. Whereby, the radiographic results shown that continued callus remodeling at week 14. A year later Boyce et al. 2009 did another experiment on the efficacy of rhBMP2 on canine model. Boyce et al. 2009 discovered that large segment of tibial defects can be effectively healed with the combination of rhBMP2 and absorbable collagen sponge or ceramic matrix at 12 week according to radiography and histology results.

1.1 Problem Statement and Objectives of the study

The use of BMP2 in birds in needed as another ongoing research in bone grafting studies for avian species and the role or effects of BMP2 in the healing process is essential in order to reduce the mortality rate of birds due to fracture with bone loss and lack of bone graft for grafting purposes. Currently, there is no established method for BMP2 application in avian veterinary services. There is also lack of biocompatible model for reference in avian research field as these BMP groups were discovered via mammals such as mouse, hamster, rabbit, goat and others (Gautschi et al. 2007). Apart from that, the suitable dosage of BMP2 to apply on the avian is still unknown and there is no standard operation procedure available for the BMP2 clinical treatment as well.

Bone Morphogenetic Protein (BMPs) have shown to induced bone formation in mammalian study. However, there is no available information on the application of avian BMP2 worldwide.

Thus, the objectives of this study are:

1. to clone and characterise the chicken-derive BMP2 gene
2. to clone and express the BMP2 protein into *Pichia* pink α-HC expression system.

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APPENDIX

>Bmp2v2 sequence

AGCGCTGTTGCTGCTACTAGATCCTTGTTGGCTTTGTTGTTGTGTAGAGTTTT
GTTGGGTGGTGCTGCTGGTTTGATGCCAGAAGTTGGTAGAAGAAGATTCTCC
GAGCCAGGTAGAGCTGCTTCTGCTGCTCAAAGACCTGAAGATTTGTTGGGT
GAGTTCGAGTTGAGACTTCTTCACATGTTCGGTTTGAAGAGAAGACCATCCC
CAGGTAAGGACGTTGTTATCCCACCATATATGTTGGACTTGTACAGATTGCAC
GCTGGTCAGCAATTGGGTTACCCATTGGAAAGAGCTGCTTCCAGAGCTAAC
ACTGTTTGTTCTTTCCACCACGAAGAGGTTTTGGAAGAGTTGCCAGAAACTT
CCGGTAAGACTGCTAGAAGATTCTTCTTCAACTTGACTTCCATCCCAAACGA
GGAATCCGTTACTTCTGCTGAGTTGCAGATCTTCAGAGAGCAAGTTCACGAG
GCTTTCGAATCCAACTCTTCCTACCACCACAGAATCAACATCTACGAGATCAT
GAAGCCAGCTACTGCTACTTCCAAGGACCCAGTTACTAGACTTTTGGACACT
AGATTGGTTCACCACAACGCTTCCAAGTGGGAATCCTTCGATGTTACTCCAG
CTGTTTTGAGATGGATCGCTCACGGTCAACCTAACCACGGTTTCGTTGTTGA
GGTTGTTCACTTGGACAAAGAGAACTCCGCTTCCAAGAGACACGTTAGAAT
CTCCAGATCCTTGCACCAGGACGAAGATTCTTGGTCCCAATTGAGACCTTTG
TTGGTTACTTTCGGTCACGACGGTAAGGGTCATCCATTGCACAAGAGAGAG
AAGAGACAGGCTAAGCACAAGCAAAGAAAGAGACACAAGTACTCTTGTAA
GAGACACCCACTTTATGTTGACTTCAACGACGTTGGTTGGAACGACTGGATC
GTTGCTCCACCAGGTTACTCTGCTTTTTACTGTCACGGTGAGTGTCCATTCCC
ATTGGCTGATCATTTGAACTCCACTAACCACGCTATCGTTCAGACTTTGGTTA
ACTCTGTTAACTCCAAGATCCCAAAGGCTTGTTGTGTTCCAACTGAGTTGTC
CGCTATCTCCATGTTGTACTTGGACGAGAACGAGAAGGTTGTTTTGAAGAAC
TACCAGGACATGGTTGTTGAGGGTTGTGGTTGTAGATAGTAAGGTACC

>|NM_204358.1| *Gallus gallus* Bone Morphogenetic Protein 2 (BMP2)

ATGGTTGCCGCCACCCGCTCCCTCCTGGCGCTGCTGCTCTGCCGGGTGCTGC
TGGGCGGCGCGGCCGGCCTCATGCCGGAGGTGGGACGGCGGCGCTTCAGC
GAACCGGGCCGCGCCGCCTCGGCCGCGCAGCGCCCCGAGGACCTCCTGGG
CGAGTTCGAGCTGCGCCTGCTCCACATGTTCGGGCTGAAGCGGCGGCCGAG
CCCCGGCAAGGACGTCGTCATCCCCCCCTACATGTTGGACCTCTATCGCCTG
CACGCCGGCCAGCAGCTGGGCTACCCGCTGGAGAGGGCCGCCAGCCGCGC
CAACACCGTGTGCAGCTTCCACCACGAAGAAGTTTTGGAAGAACTGCCAGA
AACAAGTGGGAAAACAGCACGACGTTTCTTCTTTAATTTAACTTCCATCCCT
AATGAGGAGTCTGTCACCTCAGCTGAACTCCAGATTTTTCGGGAGCAGGTG
CACGAAGCCTTTGAGAGCAACAGCAGCTACCATCACCGTATTAATATTTATG
AAATTATGAAGCCAGCCACAGCCACCTCCAAGGACCCTGTCACGAGACTTT
TGGACACCAGGTTGGTGCATCATAATGCAAGTAAATGGGAAAGTTTTGATGT
AACGCCAGCTGTTTTGAGGTGGATTGCACACGGACAACCTAACCATGGGTTT
GTGGTGGAGGTGGTTCACTTGGACAAAGAGAACAGTGCCTCCAAGAGGCA
CGTTAGGATTAGCAGGTCTTTACATCAGGATGAAGATAGCTGGTCTCAGCTC
AGGCCGTTGTTAGTGACGTTTGGGCATGATGGCAAGGGACACCCGCTCCAC
AAAAGAGAAAAGCGTCAAGCGAAACACAAACAGCGTAAGCGCCACAAATA
CAGTTGCAAAAGGCATCCGTTGTATGTGGACTTCAATGACGTGGGGTGGAAT
GACTGGATTGTTGCCCCGCCGGGGTACAGTGCCTTTTACTGCCATGGGGAAT
GTCCTTTTCCGCTGGCAGATCACCTAAACTCAACAAACCATGCCATTGTTCA
GACTTTGGTCAATTCGGTGAATTCCAAAATCCCCAAGGCTTGCTGTGTGCCG
ACAGAACTGAGTGCTATCTCAATGCTCTACCTTGATGAGAACGAAAAGGTCG
TACTAAAGAACTATCAAGATATGGTTGTGGAGGGCTGCGGGTGCCGCTG

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